

# Identification of highly brominated analogues of Q1 in marine mammals

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Capsule:

Three compounds bioaccumulated in marine mammals were identified as  $C_9H_3N_2Br_6Cl$ ,  $C_9H_3N_2Br_7$  and  $C_9H_4N_2Br_5Cl$  and are proposed to be 1'-methyl-1,2'-bipyrrole natural products.

Keywords: bioaccumulation, halogenated organic compound, high resolution mass spectrometry, halogenated bipyrrole.

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## Abstract

Three novel halogenated organic compounds (HOCs) have been identified in the blubber of marine mammals from coastal New England with the molecular formulae  $C_9H_3N_2Br_6Cl$ ,  $C_9H_3N_2Br_7$ , and  $C_9H_4N_2Br_5Cl$ . They were identified using high and low resolution electron ionization (EI) and electron capture negative ionization (ECNI) gas chromatography mass spectrometry (GCMS) and appear to be highly brominated analogues of Q1, a heptachlorinated HOC that has been suspected to be naturally-produced. These new compounds were found in Atlantic white sided dolphin (*Lagenorhynchus acutus*), bottlenose dolphin (*Tursiops truncatus*), common dolphin (*Delphinus delphis*), Risso's dolphin (*Grampus griseus*), harbor porpoise (*Phocoena phocoena*), beluga whale (*Delphinapterus leucas*), fin whale (*Balaenoptera physalus*), grey seal (*Halichoerus grypus*), harp seal (*Phoca groenlandica*) and a potential food source (*Loligo pealei*) with concentrations as high as 2.7  $\mu\text{g/g}$  (lipid weight). The regiospecificity of  $C_9H_3N_2Br_6Cl$  is suggestive of a biogenic origin. Debromination of  $C_9H_3N_2Br_6Cl$  may be significant in the formation of  $C_9H_4N_2Br_5Cl$ .

## 1. Introduction

Over the past decade, a number of previously unreported compounds have been observed to bioaccumulate in numerous organisms. These include several halogenated bipyrroles (Tittlemier et al., 1999; Vetter et al., 1999, 2000) and a selection of hydroxylated and methoxylated polybrominated diphenyl ethers (Asplund et al., 1999; Haglund et al., 1997; Malmvärn et al., 2005), none of

which are reported to be manufactured industrially. The bioaccumulated halogenated bipyrroles contain one of two bipyrrolic backbones. A heptachlorinated 1'-methyl-1,2'-bipyrrole referred to as Q1 has been reported by Vetter et al. (1999, Wu et al., 2002) and a family of structurally similar mixed halogenated 1,1'-dimethyl-2,2'-bipyrroles (DMBPs) have been identified by Tittlemier et al. (1999) (Fig. 1). These compounds have been detected in a range of marine trophic levels, including fish, seabird eggs, and marine mammals, as well as human breast milk (Tittlemier et al., 2002a; Vetter et al., 2000). In some animals their levels are comparable to industrial HOCs, including polychlorinated biphenyls (PCBs) (Tittlemier et al., 2002b; Vetter et al., 2001); however, relative concentrations are not correlated in individual animals (Tittlemier et al. 2002b). Although both types of halogenated bipyrroles are globally distributed, Q1 is more prevalent in the Southern Hemisphere (Vetter et al., 2000) and the DMBPs are more abundant in the northern Pacific Ocean (Tittlemier et al., 2002b). This suggests that the compounds are produced in specific geographical locations and subsequently transported throughout the World oceans. The toxicity of these compounds to the organisms in which they accumulate has not been well established. However in bioactivity studies, both the DMBP congeners and Q1 were shown to bind with the aryl hydrocarbon receptor and induce cytochrome P450 activity (Tittlemier et al., 2003; Vetter et al., 2004).

It has been assumed that the observed bioaccumulated halogenated bipyrroles are natural products, as no industrial sources are known. In support of their natural origin, one bioaccumulated DMBP has been shown to be at least in

part biogenic from analysis of its natural abundance radiocarbon content (Reddy et al., 2004). However, very few marine natural products have been identified with such a high degree of halogenation. An extensive review of halogenated marine natural products reported in the literature (made using the *Marinlit* database and summarized in Table 1) suggests that highly halogenated compounds are rare, with only 3% containing 6 or more halogens. Of the 1,385 known marine HOCs containing two or more halogens, only 16% are fully chlorinated. The unusual nature of Q1, with regard to the scarcity of such highly chlorinated natural products has been noted previously (Vetter et al., 1999).

While surveying for HOCs in marine mammals that had stranded on the coast of New England, we recently observed a selection of abundant unidentified halogenated compounds (UHCs) in several dolphin species. Analysis by gas chromatography high resolution mass spectrometry (GC-HRMS) revealed that three of these compounds are  $C_9H_3N_2Br_6Cl$ ,  $C_9H_3N_2Br_7$ , and  $C_9H_4N_2Br_5Cl$ . Mass spectrometry and other observations support the tentative identification of these compounds as 1'-methyl-1,2'-bipyrroles (MBPs) (Fig. 1), analogues of the heptachlorinated Q1. Here we discuss our determination of these compounds and their possible biogenic origin. Our observation of a family of structurally similar compounds, containing a more highly brominated MBP skeleton than Q1, is not unreasonable given the high proportion of halogenated marine natural products containing bromine.

## 2. Materials and Methods

### 2.1. Large scale extraction of UHCs

To determine the elemental composition of the UHCs, we used the blubber of a common dolphin (*Delphinus delphis*). This animal was provided by the Cape Cod Stranding Network, who found it fatally stranded in Westpoint, MA. The blubber was homogenized in a blender with 1:1 solution of hexane:dichloromethane (DCM), filtered, and dried over anhydrous sodium sulfate. The resulting lipids (50 g) were split into two portions and charged onto a glass column packed with Bio-beads SX-8 (BioRad Laboratories), and eluted with a 2:3 solution of hexane:DCM. The HOCs were collected in the 155 to 400 mL fraction of the eluant. The procedure was repeated until all the lipids were removed. The HOC extract was then separated on a column packed with silica gel (0.8 g) and aluminum oxide (0.5 g), eluting with hexane. PCBs were eluted in the first 5 mL. The second 5 mL fraction contained the UHCs and other HOCs. To determine the identity of the UHCs, the latter fraction was analyzed by several techniques.

To investigate the dietary contribution of UHCs to marine mammals, a potential food source was investigated. Squid (*Loligo pealei*) was provided by the Marine Resources Center at the Marine Biological Laboratory, who collected the animals from Vineyard Sound, Massachusetts. Four whole animals were homogenized with a Pro 250 handheld homogenizer (Pro Scientific Inc.), and the homogenate (275 g) was freeze dried. The dry homogenate was extracted into 9:1 hexane:methanol. The extract was washed with distilled water, dried over

anhydrous sodium sulfate, and the solvent evaporated yielding 7.12 g of lipids. The HOCs were isolated as described previously.

## 2.2. Identification of the UHCs

To identify the UHCs, low resolution gas chromatography mass spectrometry (GCMS) experiments were conducted on an Agilent 6890N series GC system connected to a 5973 network mass selective detector (70 eV ionization energy), using a J & W Scientific DB-XLB column (60 m  $\times$  0.25 mm i.d., 0.25  $\mu$ m film thickness). A temperature gradient program was used, with an initial oven temperature of 50 °C for 1 min, increased at 30 °C/min to 120 °C, then by 8 °C/min to 320°C, which was held for 30 min. The temperatures of the inlet, source, and transfer line were set at 250, 230, and 325°C, respectively. The samples were injected in splitless mode, and helium was used as the carrier gas with a flow rate of 1.1 mL/min. For the electron capture negative ionization (ECNI) GCMS experiments, methane was employed as a reagent gas and the temperatures of the source and transfer line were decreased to 70 and 250 °C, respectively. Analysis of the blubber samples were conducted in the full scan mode (50 – 750 amu). The squid extract was analyzed using single ion monitoring (SIM), scanning for the ions 575 and 577 for both UHC-1 and UHC-2 as well as the ions 697 and 699 for UHC-3.

To identify the molecular formulae of the UHCs, accurate masses for their molecular ion and fragment ions were determined by GC-HRMS. The GC-HRMS experiments were performed on a JEOL MSroute JMS-600H double focusing high

resolution magnetic sector mass spectrometer coupled with an Agilent 6890N network gas chromatograph. Compounds were separated using a Varian VF-5ms column (25 m  $\times$  0.25 mm i.d., 0.25  $\mu$ m film thickness). Perfluorokerosene was used as a calibration standard. Instrument resolution was set to 3000 at 10% peak height. Duplicate measurements were consistently within 5 ppm.

### 2.3. Quantitation of the UHCs

To provide an initial inventory and distribution of the UHCs, a variety of marine mammal samples were analyzed (Table 2). Atlantic white sided dolphin (*Lagenorhynchus acutus*), Risso's dolphin (*Grampus griseus*), grey seal (*Halichoerus grypus*), beluga whale (*Delphinapterus leucas*), and harbor porpoise (*Phocoena phocoena*) blubber samples were obtained from the Cape Cod Stranding Network and were all found stranded on the Massachusetts coast between April 2004 and July 2005. Bottlenose dolphin (*Tursiops truncatus*), fin whale (*Balaenoptera physalus*), and harp seal (*Phoca groenlandica*) samples, provided by Mystic Aquarium, were found stranded in Connecticut between March and June of 2004. Blubber samples were homogenized, and the lipids collected by filtration.

An aliquot of extracted lipid (2 g) was spiked with hexachloro 1,1'-dimethyl-2,2'-bipyrrole (DMBP-Cl<sub>6</sub>) (1.8  $\mu$ mol). We had the DMBP-Cl<sub>6</sub> custom synthesized because it is not found in environmental samples (Gribble et al., 1999) and is structurally similar to our compounds of interest. The lipids were removed as described previously. The sample was reduced in volume to 200  $\mu$ L and spiked with 6-fluoro-2,2',4,4'-tetrabromo diphenyl ether (6'-F-BDE-47) (1.0

$\mu\text{mol}$ , Chiron). The concentrations of MBP-Br<sub>6</sub>Cl, MBP-Br<sub>7</sub>, and DMBP-Br<sub>4</sub>Cl<sub>2</sub> were estimated relative to DMBP-Cl<sub>6</sub>. Each sample was injected into an HP 5890 Series II GC with a flame ionization detector (GC-FID) and separated on a CP-Sil 5CB column (60 m  $\times$  0.25 mm i.d., 0.25  $\mu\text{m}$  film thickness). Comparable relative response factors by FID have been observed for compounds with a similar number of carbon atoms (Yieru et al., 1990) and a high degree of halogenation (Erickson, 1997); hence the standard used should adequately represent the MBPs, on a molar basis. DMBP-Br<sub>4</sub>Cl<sub>2</sub> was identified by comparison of GC retention times and mass spectra to that of an authentic standard. When replicate analyses were conducted, the difference in the concentrations measured was  $< 7\%$ . Recovery of the internal standard was  $\geq 74\%$ . As a method blank, pure canola oil was extracted and analyzed by GC-FID, in which no HOCs were detected.

To test the extraction efficiency, canola oil (2 g) was spiked with 2,2',4,4',5,5'-hexachloro biphenyl (CB-153) (5.0  $\mu\text{mol}$ , Ultra Scientific), 2,2',4,4'-tetrabromo diphenyl ether (BDE-47) (2.5  $\mu\text{mol}$ , Ultra Scientific) and DMBP-Cl<sub>6</sub> (4.5  $\mu\text{mol}$ ). The lipids were removed as described above and 6'-F-BDE-47 (2.5  $\mu\text{mol}$ ) added as a recovery standard. Recoveries for PCB-153, BDE-47 and DMBP-Cl<sub>6</sub>, determined by GC-FID, were 92, 86, and 91%, respectively. All standards were of  $\geq 99\%$  purity.

#### 2.4. Investigation of the stability of the UHCs

To test the stability of the UHCs, preliminary experiments were performed. UHC extract from *D. delphis*, containing  $\sim 8 \mu\text{g}$  MBP-Br<sub>6</sub>Cl, was added to each



of eight quartz tubes. The solvent was evaporated. Sea water (1 mL) collected from Vineyard Sound, Massachusetts (salinity = 32‰ (Weber et al., 2002)) was added to six of the tubes. Distilled water (1 mL) was added to the remaining two tubes, which served as controls. The tubes were frozen in liquid nitrogen, the headspace evacuated, and the tubes sealed closed. Three tubes containing sea water and one control were placed in an oven at 70 °C in the dark for 32 days. Three tubes more tubes and one control were placed under a Philips natural sunlight fluorescent tube (negligible emission below 350 nm) for 30 days. To analyze the samples, DCM (1 mL) was added to each tube. DMBP-Cl<sub>6</sub> (1.8 µg) was added as a standard. The contents of the tube were transferred into a separatory funnel, distilled water (2 mL) added, and the UHCs extracted into 2 × 10 mL DCM. The extract was analyzed by GCMS.

### **3. Results and Discussion**

#### *3.1. Analysis of HOCs*

The HOC extract isolated from *D. delphis* was analyzed by low resolution GCMS using both EI and ECNI as ionization sources. In addition to observing many industrially-synthesized HOCs, several peaks were seen whose spectra did not correspond to any compounds reported in the literature. To better investigate these UHCs, the bulk HOC extract was separated using silica gel/Al<sub>2</sub>O<sub>3</sub> column chromatography. The UHCs eluted in a slightly more polar fraction than the PCBs and most of the 1,1-dichloro-2,2-bis(4-chlorophenyl)-ethene (*p,p'*-DDE). A GCMS-EI total ion chromatogram for the UHC fraction is shown in Fig. 2. This fraction

contained all of the extracted UHCs, and some of the bioaccumulated industrial HOCs from the previous fraction. The most abundant industrial HOCs in the UHC enriched extract were *cis* and *trans* nonachlor, 1,1-dichloro-2,2-bis(4-chlorophenyl)-ethane (*p,p'*-DDD) and *p,p'*-DDE.

### 3.2. Tentative identification of UHC-1

The most abundant UHC observed by GCMS-EI (UHC-1, Fig. 2) had an isotopic cluster resembling a compound containing one chlorine and six bromine atoms at 648 Da (Fig. 3A). This was confirmed as the molecular ion by GCMS-ECNI (Fig. 3B) for which no ions heavier than those in this ion cluster were seen. The exact molecular formula was determined by GC-HRMS. The measured accurate mass of the parent ion was compared to masses calculated for species with the following isotopic restrictions: H (0-20), C (7-14), N (0-3), Cl (0-5), Br (0-7), O (0-4), S (0-2). The elemental composition with the smallest difference between the measured and calculated masses, also containing an appropriate number of bromine and chlorine atoms to explain the observed isotopic pattern, was deemed the most probable molecular formula. A search of possible molecular formulae within  $\pm 5$  ppm of the measured molecular ion revealed 13 possibilities. Only one of these chemical formulae contained the correct proportion of halogens to account for the isotopic pattern of the molecular ion cluster. Accordingly, UHC-1 was identified as  $C_9H_3N_2Br_6Cl$ . Table 3 lists the measured and calculated masses of a selection of ions for this compound. In all cases, the fragment ion was identified as the species with the smallest difference between the measured

and calculated masses, also containing an appropriate degree of halogenation to account for the isotopic pattern of the ion cluster.

A literature search revealed no compounds with the elemental composition  $C_9H_3N_2Br_6Cl$ . Analysis of the compound by high and low resolution GCMS yielded some information about the chemical structure. The predominant fragment ions resulted from loss of  $Br^\bullet$ ,  $Cl^\bullet$  and  $CH_3^\bullet$ . Observation of a methyl group accounts for all of the hydrogens in this compound. The low ratio of carbon to other atoms suggests a high degree of saturation. Following these observations, we propose the most reasonable structure for  $C_9H_3N_2Br_6Cl$  is chloro hexabromo 1'-methyl 1,2'-bipyrrole (Fig. 1, MBP- $Br_6Cl$ ). There are five possible isomers of MBP- $Br_6Cl$ . However when the UHC extract was analyzed with two capillary columns of different polarity (DB-XLB and DB-5MS), only one peak was observed for this compound using GCMS. This suggests that only one isomer is present. The absence of other isomers suggests a highly regioselective or regiospecific synthesis of these compounds. This degree of selectivity is suggestive of natural production. There currently insufficient information to identify the location of the chlorine atom. In the mixed halogenated DMBPs reported by Tittlemier et al. (2002a), chlorination of the carbon adjacent to the N- $CH_3$  group is exclusively observed; this pattern may also be observed for MBPs. This position is known to be the most reactive to electrophiles in monopyrroles (Jones, 1990), and the regiospecificity may reflect a degree of electrophilic character in the chlorination mechanism of halogenated bipyrroles. The most effective method for identifying the specific isomer of MBP- $Br_6Cl$  would be to isolate a sufficient

quantity to obtain nuclear magnetic resonance (NMR) spectra and conduct an X-ray crystallographic analysis. This will require isolation of a considerable amount of material. The identity of the compound may be verified by synthesis of an authentic standard and comparison of mass spectra and relative GC retention times (Teuten et al., 2005a).

### 3.3. Determination of UHC-2 and UHC-3

Similar procedures identified UHC-2 and UHC-3. UHC-2, an apparently monochlorinated pentabrominated compound with a molecular ion at 570 Da, was determined to be  $C_9H_4N_2Br_5Cl$ . UHC-3, which has an isotopic pattern characteristic of a heptabrominated compound and molecular ion at 691 Da, was identified as  $C_9H_3N_2Br_7$ . Calculated and measured masses for the molecular ions and prominent fragment ions for UHC-2 and UHC-3 are listed in Table 3. A literature search indicated no known compounds with these molecular formulae. As with UHC-1, the MS-EI spectra of these compounds show predominant loss of halogens (Fig. 4). Further similarities between the mass spectra of these three compounds suggest they have similar molecular structures. We propose that the structures of  $C_9H_3N_2Br_7$  and  $C_9H_4N_2Br_5Cl$  are MBP- $Br_7$  and MBP-H $Br_5Cl$ , respectively (Fig. 1).

All three UHCs show a fairly abundant doubly charged  $[M-2Br]^{2+}$  ion. These peaks show the same halogen isotopic pattern as the singly charged ions, but the peaks are only separated by 1 Da. For UHC-1 and UHC-3, this peak is of a comparable intensity to that of the molecular ion. Abundant  $[M-2Br]^{2+}$  ions

were also observed for DMBPs (Tittlemier et al., 1999). However, the compounds identified in this report are clearly not DMBPs, as the molecular formulae determined from the accurate masses contain an insufficient number of hydrogens to furnish two methyl groups. Additionally, GC-HRMS experiments identified the ions  $C_4BrCl^+$  and  $C_4Br_2^+$  for UHC-1 and UHC-3, respectively. These are interesting fragments arising from either the non-methylated pyrrole ring or from a rearrangement of the methyl group into the N-methyl pyrrole ring (Jones, 1990) with subsequent fragmentation. This fragmentation does not appear to have been reported for monopyrroles. The 1'-methyl 1,2'-bipyrrole system is uncommon and its typical mass spectrometry has not been discussed in the literature. A similar ion is seen for UHC-2 at 84 Da, which is consistent with  $C_4HCl^+$  including the isotopic pattern. An accurate mass was not determined for this ion, so this identity cannot be definitively assigned. These proposed structures contain the same bipyrrole skeleton as Q1, which also accumulates in the blubber of marine mammals. When examining the GCMS-EI spectrum for Q1 published in the literature (Vetter et al., 1999) an ion was seen at 118 Da, with an isotopic distribution suggestive of two chlorines. It is possible that this peak corresponds to  $C_4Cl_2^+$ , supporting a structural similarity between the compounds identified here and Q1.

### 3.4. Quantitation of the UHCs

The concentrations of MBP- $Br_6Cl$  and MBP- $Br_7$  in a selection of marine mammals found stranded on the coasts of Massachusetts and Connecticut were

estimated using GC-FID and are listed in Table 2. For reference, the concentration of one of the most abundant PCBs in the samples, CB-153, is also reported. In all cases the concentration of CB-153 is higher than that of the MBPs. No correlation between the MBP concentrations and CB-153 were seen. Although it appears that the MBPs may be more abundant in dolphin than other species of marine mammals, it must be noted that the sample selection includes adult dolphins but only juvenile animals of other species. Recorded trends for HOCs in marine mammals show higher amounts in mature than in immature animals (Borgå et al., 2004). Thus, higher MBP concentrations in dolphins may be a consequence of increased accumulation with age.

To investigate the mode of transfer of the MBPs to marine mammals, a potential dietary component was examined. Squid are known to be a preferred food source of common and white-sided dolphins in the New England area (Weisbrod et al., 2000). Analysis of the squid HOC extract by GCMS-ECNI using SIM showed the presence of all three MBPs discussed herein. This indicates that these compounds bioaccumulate in dolphins, and likely other marine mammals, as a result of dietary exposure. Stable nitrogen isotope analysis of whole tissues has revealed that structurally similar DMBPs are present at a range of trophic levels (Tittlemier et al., 2002a) and further analysis may indicate the same phenomenon for MBPs.

A screening for other halogenated bipyrroles known to accumulate in marine mammal blubber revealed DMBP-Br<sub>4</sub>Cl<sub>2</sub> in some animals (Table 2). Several dolphin samples also contained a small peak whose spectra appeared to

match that of Q1, although this identity could not be confirmed due to lack of an authentic standard.

To illustrate the potential for the MBPs to bioaccumulate, estimated vapor pressures and octanol-water partition coefficients ( $K_{ow}$ ) were compared with those of industrial HOCs. Using U.S. Environmental Protection Agency EPI (Estimation Programs Interface) Suite software, the vapor pressures and  $K_{ow}$  were estimated at  $0.1 \times 10^{-6}$  to  $2 \times 10^{-6}$  Pa and  $> 10^7$ , respectively. It is worth noting that when a similar approach was used for the DMBPs, for which the vapor pressure and  $K_{ow}$  have been experimentally determined (Tittlemier et al. 2004a), this software over-estimated  $\log K_{ow}$  by an order of magnitude and under-estimated the vapor pressure by approximately two orders of magnitude. The difference between the estimated and experimentally determined values for the DMBPs suggests similar errors may be observed for the MBPs. Nevertheless, the estimated values indicate a tendency for these compounds to bioaccumulate and are similar to values for PCBs with  $\geq 5$  chlorines.

### *3.5. Origin and distribution*

Although these compounds have not been reported previously, it is possible they have gone undetected in samples from other locations. They may have been missed either because they were not recognized, or because they were unstable under the conditions used to remove the lipids. Early attempts in this study to isolate the UHCs by saponification or acid degradation of the lipids resulted in loss of the MBPs. Interestingly, Q1 did not show significant reaction with

concentrated sulfuric acid (Vetter et al., 1999). The higher reactivity of the brominated MBPs compared to Q1 likely arises because bromine is a better leaving group than chlorine.

It has been suggested previously that bioaccumulated bipyrrroles are produced naturally (Reddy et al., 2004; Wu et al., 2002). The presence of radiocarbon in one bioaccumulated DMBP implies, at least in part, a biogenic origin (Reddy et al., 2004). Additionally, a structurally similar hexabrominated 2,2'-bipyrrrole is a known marine bacterial metabolite (Andersen et al., 1974). However, there are no known sources of MBPs, either natural or industrial. While it is possible that these MBPs are unreported byproducts of industrial processes, their structural similarity to biogenic compounds suggests that they are produced naturally by an unidentified marine organism. Work is underway to isolate sufficient amounts of these compounds for analysis of their natural abundance radiocarbon content, which will allow us to distinguish between a natural and an industrial source (Reddy et al., 2002; Teuten et al., 2005b). Stable isotope analysis of the individual compounds will likely also provide insight into their source. Analysis of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  may identify the carbon source and the trophic level of the organism that originally synthesized the compounds (McClelland and Montoya, 2002).

A large number of halogenated natural products have been identified (Gribble, 2003), including some that resemble persistent, anthropogenic contaminants (Carté and Faulkner 1981; Utkina et al., 2001, 2002). As discussed in the introduction, very few highly halogenated marine natural products are



known, and of these only one of these contains a bipyrrrole moiety (Andersen et al., 1974). However, it must be noted that this record is far from complete, as newly discovered halogenated natural products are reported on a regular basis. As illustrated in Table 1, a small proportion (16%) of the known natural HOCs containing two or more halogens are fully chlorinated. This may seem unexpected, as chloride is ~300 times more abundant in seawater than bromide (Pilson, 1998). The preference for bromination is likely due to the lower oxidation potential of bromide (Price et al., 1988), resulting in its more facile incorporation into organic compounds by halogenase enzymes. In light of this, the heptachlorinated nature of Q1 is unusual. The existence of a family of structurally similar compounds, containing a more highly brominated MBP skeleton than Q1 seems reasonable given the high proportion of halogenated natural products containing bromine. Interestingly, there are a range of other similar compounds containing different proportions of chlorine and bromine that were not present in our samples, including MBP-Br<sub>5</sub>Cl<sub>2</sub>, MBP-Br<sub>4</sub>Cl<sub>3</sub>, MBP-Br<sub>3</sub>Cl<sub>4</sub>, MBP-Br<sub>2</sub>Cl<sub>5</sub> and MBP-BrCl<sub>6</sub>. It is possible that these compounds will also eventually be found in biological samples.

It is plausible that more highly chlorinated MBPs form by halide exchange reactions, in which the equilibrium is shifted toward an increase in chlorination, due to the higher concentration of chloride in seawater relative to bromide. Marine halogen exchange reactions occur primarily by light-activated radical reactions or nucleophilic exchange (Ballschmiter, 2003). To probe the possibility of halide exchange reactions occurring by these two mechanisms on the MBPs

extracted from *D. delphis*, solvent-free UHC extract was mixed with seawater and exposed to either heat or light at natural sunlight wavelengths. No halide exchange was observed for a sample stored at 70 °C in the dark for 32 days, or after 30 days of continuous exposure to simulated sunlight. These results do not support formation of more highly chlorinated MBPs by simple halogen exchange reactions.

Biological transformations of the MBPs are also possible. For example, debromination of MBP-Br<sub>6</sub>Cl could generate MBP-HBr<sub>5</sub>Cl. Debromination of other HOCs, including polybrominated diphenyl ethers (PBDEs) and hexabromobenzene, has been observed in carp (Stapleton et al., 2004) and rats (Yamaguchi et al., 1988), respectively. Various microbial dehalogenation reactions are known (Janssen et al., 2001). As discussed above, only one isomer of MBP-Br<sub>6</sub>Cl was detected in the samples examined. Loss of one bromine atom from this compound could result in the formation of four to six possible isomers of MBP-HBr<sub>5</sub>Cl. After close inspection of eight samples listed in Table 2 using GCMS-ECNI, we found three additional compounds that we have tentatively identified as isomers of UHC-2 on the basis of their mass spectra. Assuming a comparable response factor for all four of these compounds, UHC-2 was at least 10 times more abundant than the other isomers in all the samples. Nevertheless, this suggests formation of MBP-HBr<sub>5</sub>Cl by a regioselective, but not regiospecific, debromination of MBP-Br<sub>6</sub>Cl. If indeed MBP-Br<sub>6</sub>Cl is a natural product, it would have been in the environment long before the manufacture and release of industrial HOCs beginning in the 1920s (Stringer and Johnston, 2001). A number of ubiquitous industrial

HOCs are known to undergo microbial dehalogenation reactions (Smidt and de Vos, 2002). It is possible that the environmental presence of MBPs may have helped organisms to develop the pathways by which they dehalogenate structurally similar industrial HOCs, such as PCBs.

Limited knowledge surrounding the bioactivity of halogenated bipyrrroles suggests that the compounds we have reported herein may induce a biological response (Tittlemier et al., 2003; Vetter et al., 2004). The potential human health implications are unknown. Halogenated DMBPs have been observed in a variety of fish and seafood available for commercial retail throughout Canada, indicating exposure of humans to these compounds (Tittlemier, 2004b). Human exposure to halogenated bipyrrroles is also apparent through detection of Q1 in the breast milk of women from the Faroe Islands (Vetter et al., 2000). These observations suggest that more detailed work should be conducted on the exposure of humans to halogenated bipyrrroles and the implications of such exposure. Last, if truly naturally produced, these compounds have likely been present in the environment for a much longer time period than industrially-synthesized HOCs. Therefore, these compounds may be excellent subjects to study the long-term fate of HOCs with similar physical properties, such as PCBs.

#### **4. Conclusions**

In summary, three novel bioaccumulated compounds have been identified in the blubber of marine mammals from New England. These compounds have been identified as  $C_9H_3N_2Br_6Cl$ ,  $C_9H_3N_2Br_7$  and  $C_9H_4N_2Br_5Cl$  by GC-HRMS. The

evidence supports these three compounds having a 1'-methyl 1,2'-bipyrrole skeleton, identical to that of the heptachlorinated MBP, Q1. This suggests the existence of a family of MBPs with different halogenation patterns, akin to the DMBPs reported by Tittlemier et al. (1999). A tendency for the MBPs to accumulate in various species of marine mammals, particularly dolphins, has been observed. Our identification of MBP-HBr<sub>5</sub>Cl represents the first report of a bioaccumulated bipyrrole whose rings are not fully halogenated.

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## Figures Captions

Fig. 1. Halogenated bipyrroles bioaccumulated in the marine food web. Q1 was identified by Vetter et al. (1999) and the DMBPs by Tittlemier et al. (1999).

Fig. 2. GCMS-EI chromatogram of the UHC fraction of blubber extract from *D. delphis*. The prevalent, unlabelled peaks are HOCs whose identification is in progress. The PCBs and most of the *p,p'*-DDE have been removed from this sample.

Fig. 3. GCMS spectra of UHC-1 (MBP-Br<sub>6</sub>Cl), using (A) EI and (B) ECNI.

Fig. 4. GCMS-EI spectrum of (A) UHC-2 (MBP-HBr<sub>5</sub>Cl) and (B) UHC-3 (MBP-Br<sub>7</sub>).

## Tables

Table 1.

Distribution of halogens in known marine halogenated natural products. A preference for polyhalogenated compounds to contain exclusively bromine or a combination of different halogens is observed. The data in this table was compiled using the *MarinLit* database (February 2005 update).

	# halogens								
	1	2	3	4	5	6	7	8	11
# fully chlorinated natural products	544	82	51	30	13	45	0	0	2
# fully brominated natural products	601	406	107	112	21	23	1	3	0
# fully iodinated natural products	36	11	3	2	0	0	0	0	0
# mixed halogenated natural products	-	188	155	81	37	12	0	0	0

Table 2.

Estimated concentrations of HOCs in a selection of marine mammals stranded in New England, listed in order of decreasing trophic status (Pauly et al., 1998).

animal	Details <sup>a</sup>	Concentration (µg/g lipid) <sup>b</sup>						Accession no.
		MBP-Br <sub>6</sub> Cl	MBP-Br <sub>7</sub>	MBP-HBr <sub>5</sub> Cl	DMBP-Br <sub>6</sub> Cl <sub>2</sub>	Q1	CB-153	
Risso's dolphin ( <i>G. griseus</i> )	m, j.	1.7	0.4	+	+	+	2.7	CCSN05-188Gg
Risso's dolphin ( <i>G. griseus</i> )	m, a	1.0	0.1	+	+	+	1.5	CCSN05-192Gg
Common dolphin ( <i>D. delphis</i> )	m, a	2.7	1.1	+	0.5	+	7.8	CCSN04-177Dd
Common dolphin ( <i>D. delphis</i> )	f, j	1.2	1.6	+	-	-	2.3	CCSN04-219Dd
Common dolphin ( <i>D. delphis</i> )	m, j	0.4	0.5	+	+	-	0.7	CCSN04-218Dd
Bottlenose dolphin ( <i>T. truncatus</i> )	f, a	2.1	1.1	+	-	-	15.8	SxTt0410
Atlantic white sided dolphin ( <i>L. acutus</i> )	m, a	1.9	0.7	+	0.2	+	8.4	CCSN04-116La
Atlantic white sided dolphin ( <i>L. acutus</i> )	f, a	2.5	2.7	+	-	-	3.9	CCSN05-040La
Harbor porpoise ( <i>P. phocoena</i> )	m, j	0.2	0.2	+	-	-	0.8	CCSN04-030Pp
Gray seal ( <i>H. grypus</i> )	m, j	0.3	0.03	+	-	-	5.0	CCSN04-186Hg
Beluga whale ( <i>D. leucas</i> )	m, j	0.1	0.01	+	+	-	0.9	04513-DI
Harp seal ( <i>P. groenlandica</i> )	m, j	0.04	0.005	+	-	-	0.1	SyPg04180314
Fin whale ( <i>B. physalus</i> )	m, j	0.2	0.1	+	-	-	1.1	SyBp0448
Squid ( <i>L. pealei</i> )		0.002	0.007	+	-	-	0.1	

<sup>a</sup> m = male, f = female, a = adult, j = juvenile, n.a. = not available. <sup>b</sup> - = not detected, + = detected by GCMS, but not quantified (concentrations were either below the GC-FID detection limit or co-eluted in the GC-FID analysis).

Table 3.

Measured and calculated masses for the molecular ions and fragment ions of UHC-1, UHC-2, and UHC-3 detected in *D. delphis* blubber, determined by GC-HRMS.

Compound	Ion	Fragment lost	Measured mass (Da)	Calculated mass (Da)	Difference (ppm)
UHC-1	$C_9H_3N_2Br_6Cl^+$	-	647.5084	647.5085	0.2
	$C_9H_3N_2Br_6^+$	$Cl^\bullet$	612.5381	612.5396	-2.5
	$C_9H_3N_2Br_5Cl^+$	$Br^\bullet$	568.5895	568.5902	-1.2
	$C_9H_3N_2Br_4Cl^+$	$2Br^\bullet$	489.6738	489.6718	4.1
	$C_9H_3N_2Br_3Cl^+$	$3Br^\bullet$	410.7537	410.7535	0.6
	$C_8N_2Br_2^{81}BrCl^+$	$3Br^\bullet$ , $CH_3^\bullet$	397.7301	397.7280	5.3
	$C_9H_3N_2Br^{81}BrCl^+$	$4Br^\bullet$	333.8336	333.8331	1.5
	$C_4BrCl^+$		161.8870	161.8872	-1.1
UHC-2	$C_9H_4N_2Br_4^{81}BrCl^+$	-	571.5953	571.5959	-1.0
	$C_9H_4N_2Br_4^{81}Br^+$	$Cl^\bullet$	536.6246	536.6270	-4.5
	$C_9H_4N_2Br_4Cl^+$	$Br^\bullet$	490.6785	490.6796	-2.2
	$C_9H_4N_2Br_3Cl^+$	$2Br^\bullet$	411.7620	411.7613	1.7
	$C_9H_4N_2Br_2Cl^+$	$3Br^\bullet$	322.8421	332.8430	-2.7
UHC-3	$C_9H_4N_2Br_4^{81}Br_3^+$	-	697.4524	697.4518	0.8
	$C_9H_4N_2Br_6^+$	$Br^\bullet$	612.5392	612.5396	0.7
	$C_9H_4N_2Br_4^{81}Br^+$	$2Br^\bullet$	535.6180	535.6192	-2.3
	$C_9H_4N_2Br_4^+$	$3Br^\bullet$	454.7015	454.7029	-3.1
	$C_8N_2Br_3^{81}Br^+$	$3Br^\bullet$ , $CH_3^\bullet$	441.6767	441.6774	-1.7
	$C_9H_4N_2Br_2^{81}Br^+$	$4Br^\bullet$	377.7837	377.7826	2.9
	$C_4Br^{81}Br^+$		207.8372	207.8346	2.6